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Original Research

Phytochemical properties of Iranian organic saffron stigma: antioxidant, anticancer and apoptotic approaches

M. A. Behdani[1], R. Hoshyar[2,3*]

[1] Saffron Research Group, University of Birjand, Birjand, Iran
[2] Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran
[3] Department of Biochemistry, School of Medicine, Birjand University of Medical Sciences, Birjand, Iran

Abstract: Agronomic and environmental factors affect quality and quantity of constituents in Saffron. In this study, we compared chemical and antioxidant compounds of organic (OS) and inorganic (IOS) stigma of saffron and evaluated their anti-proliferative and apoptosis effects on cancer cells. Total antioxidant capacity of both saffron were characterized by FRAP, DPPH and Folin-Ciocalteu. HPLC and MTT methods were used to assay the amount of their secondary metabolites and anticancer effects, respectively. The expression of two apoptosis-related genes in treated cells evaluated by quantitative Real Time-PCR analysis. Our data indicated that OS has more secondary metabolites, antioxidant and cytotoxic properties compared to IOS. OS significantly inhibited cell viability in a dose- and time- dependent manner. Herb-induced apoptosis associated with increased expression of Bax and decreased Bcl2 gene leading eventually to a time-dependent increase in Bax/Bcl-2 ratio. Therefore, we can suggest organic saffron has promising and selective inhibitory effects on cancer cell proliferation.

Key words: Anticancer, antioxidant, apoptosis, HPLC, saffron.

Introduction

Plants produce secondary metabolites to aid their growth and development including defense, coloring and symbiosis (1, 2). Also these herbal components have various medicinal properties such as anti-lipidemia, anti-tumor, anti-viral and anti-ageing activities (3). Analysis of secondary metabolites revealed their distribution is highly diverse (4). So, it seems that a plant has subset of species-specific secondary metabolites.

Saffron (Crocus sativus L.) is one of the oldest medicinal crops in Iran that farmers throughout its agro-history had the largest role for development and its transition into a new era (5, 6). The stigma of saffron has many pharmacological usages including anti-oxidant, anti-inflammatory and anti-cancer due to its active molecules (7, 8). Chemical composition analyses illustrated that primary metabolites contain the most composition of saffron and the fewer amount of secondary metabolites was found

. Crocins, picrocrocin, and safranal are main bioactive compounds of stigma that are responsible for its color, flavor, and aroma, respectively (9).

Production, processing and consumption of saffron have grown based on local knowledge and ecological perspectives (10). In saffron cultivation areas, other plant cannot be completely eco-friendly, economically justified and socially fair. Studies have shown that saffron because of its cultivation and production methods has high potential to be produced as an organic production, in comparison with many other agricultural products (11). However ranges of all secondary metabolites can vary greatly due to different growing conditions, the original place, cultivation practices and drying and packing processes (12, 13).

Here, we compared the percentage of chemical compounds in organic and inorganic saffron types and then evaluated their anti-cancer and apoptotic activities in different cancers for instance breast, brain and stomach.

Materials and Methods

Preparation of saffron extracts

Saffron collected from farms with the requirements of organic production in Qaen, Iran. When the organic and inorganic conditions of the plantation field validated, the aqueous extract prepared according to our previous work (14).

Ferric Reducing Antioxidant Power (FRAP) assay

The total antioxidant activity (TAA) of saffron extracts was determined by FRAP assay. The results expressed in M Fe (II)/g DW of plant extracts (15).

Folin-Ciocalteu assay

The total phenolic contents (TPC) of herbal extracts were measured using the Folin–Ciocalteu method. The data were expressed as milligram Gallic acid equivalents (GAE)/g DW of plant extracts (16).

DPPH radical- scavenging activity

The percentage of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenges ability of saffron was reported (%; 17).

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*** Corresponding author:** Reyhane Hoshyar, M.Sc., Ph.D., Birjand University of Medical Sciences, P.O. Box: 9717853577, Birjand, Iran. Email: reyhaneh.houshyar@gmail.com or hooshyar@bums.ac.ir

High-performance liquid chromatography (HPLC) assay

For HPLC determination, saffron stigmas powder (20 mg) suspended in 1 ml of methanol–water (50:50, v/v) and magnetically was stirred during 24 h at 4° C in the dark. After extraction, samples centrifuged at 30,000g for 20 min to eliminate plant residues and then the supernatant were collected. Before quantitative chromatographic analysis, 1 ml of 2-nitroaniline (0.5 mg/ml) was added as an internal standard to 1 ml of each tested sample. Each sample of the dehydrated stigmas (50 µl) was ground with a potter using double distilled water (500 ml) containing acetonitrile and methanol (as mobility phase) for 60 min at room temperature. Quantification carried out at 250, 310 and 440 nm for picrocrocin, safranal and crocin, respectively. HPLC analysis was performed on a Shimadzu Shim-pack C18 VPODS column equipped with a binary pump, a multiple UV wavelength photo-diode array detector, linked to a computer system. Finally, all the chromatographic data were processed using Shimadzu GC Solution Empower Software (version 2).

Cell culture and Cell viability assay

Different human cancer (MDA-MB-468 (breast), AGS (stomach(, U87 (brain)) and normal epithelial (MCF10-A) cell lines were provided from Iranian Biological Resource Center (IBRC). Cancer and normal cells were kept in RPMI-1640 and DMEM: Ham's F-12 mediums, respectively. Each medium was supplemented with 10% FBS serum, 5% penicillin and streptomycin 50µg/µL solution in culture flasks at 37°C in 5% humidified CO2 incubator. The cells were treated with different concentrations of OS and IOS (0.5, 1, 1.5and 3 mg/ml) at various time intervals (0- 72 hours). Their cytotoxic effects were evaluated by MTT assay and the IC50 value of herbs calculated using the dose- and time-dependent curves by linear interpolation (18).

Quantitative Real-Time PCR

Apoptotic effects of IOS (3 mg/ml) on cancer cells (U87) studied by measuring Bax/Bcl2 ratio in mRNA level as apoptotic index. The Quantitative RT-PCR for Bax and Bcl2 was carried out using previous primers (19).

Statistical analysis

Results are expressed as the means ± SEM of at least three independent experiments (n=3). Data were analyzed using one-way ANOVA and with Tukey's post hoc test to assess differences between experimental groups. Statistical significances were inferred at P≤0.05, (PRISM 5.0; Graph- Pad Software Inc.).

Results

Aantioxidant assay

Our data indicated that total antioxidant and phenolic values of the OS extract is significantly higher compared to IOS extract (P<0.05, Table 1). The results of DPPH assay showed that various concentrations of (1.25, 2.5 and 5 mg/ml) OS exhibited more free radical scavenging activity than similar concentrations of IOS extracts, dramatically (P<0.05, Figure 1).

Table 1. The total antioxidant activity and total phenolics of both OS and IOS extracts.

Antioxidant Contents	OS	IOS
TAA[1] (MFe(II)/g DW)	480±6.69*	395±7.17
TPC[2] (GAE/g DW)	320±3.25*	265±5.15

Data were mean ± SEM (n=3). *P<0.05 Antioxidant contents of OS (2.5 g/l) in comparison with IOS (2.5 g/l).[1]Total antioxidant activity expressed in M Fe (II) per g DW of plant extracts.[2]Total phenol content expressed in mg of Gallic acid equivalents (GAE) per g DW of plant extracts (One-way ANOVA followed by Tukey's post hoc test).

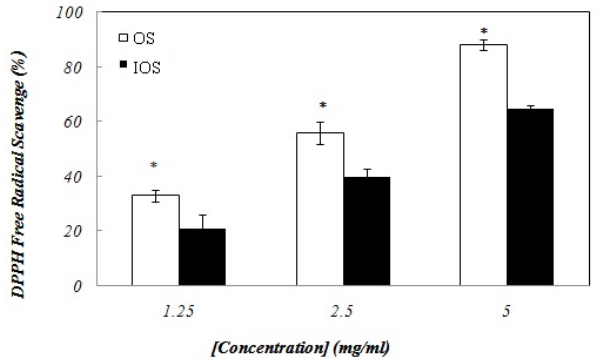

Figure 1. Percentage of DPPH radical quenching activity of OS and IOS extracts (2.5 mg/ml). Data are expressed as mean ± SEM (n=3).*P<0.05 DPPH %of different concentrations (1.25, 2.5 and 5 mg/ml) OS in comparison with IOS (One-way ANOVA followed by Tukey's post hoc test).

HPLC

Analysis of HPLC chromatograms of saffron extracts of different origins allowed to identifying different peaks, belonging to components of saffron. As presented in Table 2, OS extract had more amounts of crocin, picrocrocin and safranal compared to IOS, significantly (P<0.01).

MTT assay

As Shown in Figure 2 the viability of treated cancer cells (0-3 mg/ml) was markedly reduced in dose- and time-dependent manner compared to untreated cells after different incubations (0-72 hours, P<0.05). The inhibitory effect of OS extract on cancer cell proliferation was superior to that of IOS extract. Also the IC50 values of both extracts of saffron after different incubations for various cancer cells are reported in Table 3. The parallel treatments of the normal cells with these herbal extractions demonstrated a much less inhibitory effect on the viability of MCF-10A cells (Figure 3).

Table 2. Mean of quantified data of HPLC results demonstrating the amount of some components of saffron samples (mg of component per 1 g of sample).

Samples	Crocin (mg/g)	Picrocrocin (mg/g)	Safranal (mg/g)
OS	15.5 ± 0.14**	11.09 ± 0.33**	6.66 ± 0.21**
IOS	13.04 ± 0.09	9.47 ± 0.47	5.14 ± 0.11

Data were mean ± SEM (n=3). **P<0.01secondary metabolites of OS in comparison with IOS. (One-way ANOVA followed by Tukey's post hoc test).

Table 3. IC_{50} (mg/ml) values of both extracts of saffron against various cancer cells after 24-72h incubations.

Cancer Cell Line	Samples (mg/ml)	24 h	48h	72h
MDA-MB-468	OS	3±0.5*	1.5±0.8*	0.8±0.2*
	IOS	4±0.7	2±0.7	1.1±0.5
AGS	OS	3.2±0.5*	1.8±0.7*	1±0.3*
	IOS	5±0.7	2.5±0.5	1.3±0.8
U87	OS	3.1±0.3*	1.6±0.8*	1.1±0.2*
	IOS	4.5±0.8	2.1±0.3	1.3±0.5

Data were mean ± SEM (n = 3). *P<0.05 IC50 of OS in comparison with IC_{50} of IOS for various cancer cells at different time treatments (One-way ANOVA followed by Tukey's post hoc test).

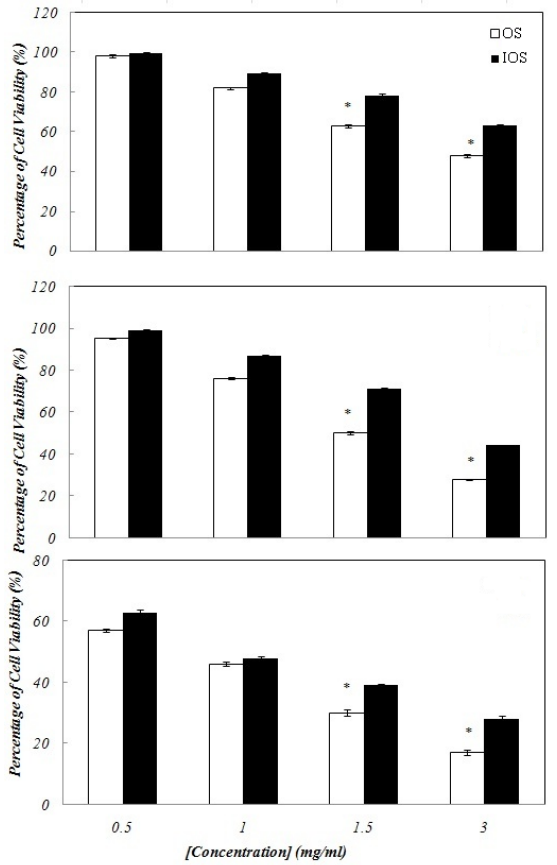

Figure 2. The cytotoxic effect of different concentrations of OS and IOS (0.5, 1. 1.5 and 3 mg/ml) on MDA-Mb-468 cell line after 24 (up), 48 (middle) and 72 hours (down) incubations. Data were mean ± SEM (n = 3). *P<0.05cytotoxic effect of OS (1.5 and 3 mg/ml) in comparison with cytotoxic effect of IOS (1.5 and 3 mg/ml) at different time treatments (One-way ANOVA followed by Tukey's post hoc test).

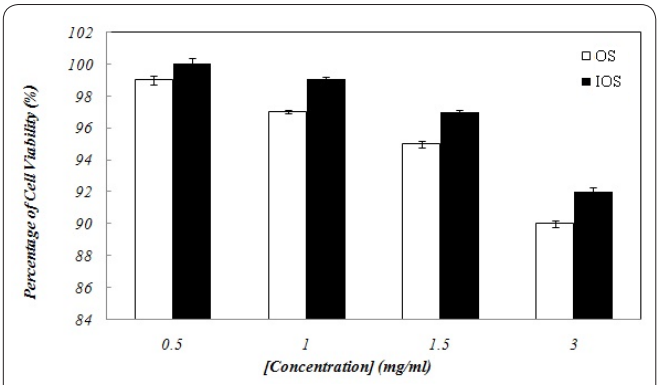

Figure 3. The cytotoxic effect of different concentrations of OS and IOS (0.5, 1. 1.5 and 3 mg/ml) on MCF-10A cell line after 48 hours incubation.Data were mean ± SEM (n = 3) (One-way ANOVA followed by Tukey's post hoc test).

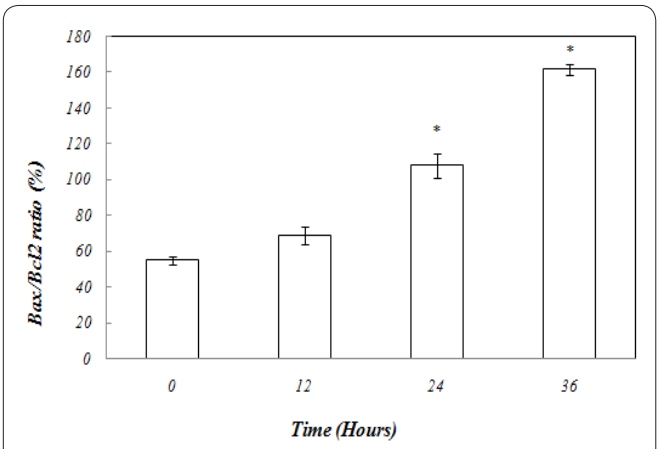

Figure 4. Apoptotic effects of both types of saffron (3 mg/ml) on Bax/Bcl-2 ratio in U87 cells. Data represented relative gene expression (Target/bactin) (One-way ANOVA followed by Tukey's post hoc test).

qRT-PCR

Quantitative real-time PCR results showed that IOS (1.3±0.5 mg/ml) significantly increased Bax/Bcl-2 ratio in treated cancer cells in a time depended manner (Figure 4).

Discussion

Saffron is cultivated in a wide range of environments with mild to dry climates (6). The different environmental and cultivation conditions cause considerable alteration in chemical composition of saffron (20). This herb historically has been used from organic agronomic practices such as nutrition according to organic fertilizers, non-chemical methods for pests and weeds management and use of labor work for its production and processing. Also its production in Iran is largely based on the principles methods of organic farming both in terms of technology and social aspects. However, such products of saffron cannot be recognized as organic in international markets due to strict certification process required for organic products. Therefore, currently a small proportion of saffron is certified organically, but this will be much more of attention in the future. The effective methods such as TLC, HPLC and microscopic analysis lead to discriminate different types of saffron (21). While the mentioned discriminating methods were determined several compounds, specific to OS and IOS, there is no comparative study on these two types, yet. In this study for first time, a complete picture of antioxidant capacity of both types of saffron were indicated and compared via FRAP, Folin-Ciocalteu and DPPH

analysis. Our results were demonstrated more antioxidant, phenolic compounds and higher free radical scavenging property in OS than IOS, significantly. The high antioxidant activity of herbs is primarily due to the high levels of phenolic and caratenoid content and our observations are consisting with previous studies (14, 22). In addition, HPLS data were illustrated OS extract had more amounts of active metabolites such as crocin, picrocrocin and safranal when compared to IOS, significantly ($P<0.01$). In this context, the data of Ibrahim (23) indicated that the use of organic condition can enhance the production of secondary metabolites and improve antioxidant activity of herb. It might be suggested that if pharmalogical researches use organic components they can make real drugs safer and more effective for different types of diseases.

Differential analysis of saffron chemo-biological characteristics along with previous facts about it antitumor effects (24, 25) were compelled us to assess the cellular effects of OS and IOS. To this aim, we were designed an MTT experiment to evaluate the antiproliferative of both types of saffron on three cancers breast, stomach and brain cells. The data significantly were showed that OS extract (1.5 and 3 mg/ml) is more capable to arrest cancer cell growth compared to IOS after 24-72 hours incubations ($P<0.05$). However at low concentrations (0.5 and 1 mg/ml) it had no such effect. The IC50 values strongly were indicated that the effective doses of OS extracts were lower compared to IOS extracts after different incubation times (0–72 h, Table 3). The parallel treatments of the normal cells with these herbal extractions were demonstrated a much less inhibitory effect on the viability of MCF-10A cells. The cytotoxic effect of OS extract may be related to its higher content of phenolic and carotenoid components.

Numerous studies have also suggested that saffron exert potent anti-carcinogenic effects due to apoptosis induction (24, 26, 27). Apoptosis is regulated by several mediator genes such as proapoptotic Bax and antiapoptotic Bcl2 genes, which are important targets for cancer therapy. It was revealed that crocin from saffron induced apoptosis in cancer cells through high Bax/Bcl-2 ratio. Our data also were in agreement with previous results.

Therefore, our results indicated stronger antioxidant effects because more of its phyto-constituents including phenolic and carotenoid compounds in OS extract. The plenolics have been identified as anti-proliferative agents due to their ability to cell cycle arrest, induce apoptosis and destruction mitotic spindle formation (28,29). On the other hand, these components effectively inhibited functional enzymes in cancer pathologies including 5-lipoxygenase and cyclo oxygenase also modulated the several proteins that involved in cancer promotion for instance protein kinases, epidermal growth factor receptors, and cyclin-dependent kinases (28-30). We found a higher correlation between total phenolics and anticancer property in OS sample.

In conclusion, the current comprehensive analysis, for the first time, reported the strong evidence that OS has superior biological features compared to IOS. It suggests that cultivation and growth conditions of saffron are better to be developed in organic farms to use it as an alternative cancer treatment.

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